

♂ Gonorrhea $\Rightarrow$ ^{Can be} Diagnosed
by only microscope
 $\Rightarrow$ Gram -ve

Define Colony?

DNA probe?
single stranded DNA/RNA
Complementary to specific organism

Define serological diagnosis

Define serological Identification

PRACTICAL MICROBIOLOGY

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Chapter 24

LABORATORY DIAGNOSIS OF INFECTIOUS DISEASES

The laboratory diagnosis of infectious diseases involves 2 main diagnostic methods:

- I) **Direct method**: which depends on the detection of microorganisms, their structural components or their products in specimens collected from the patient (e.g. urine, blood, sputum, CSF.....etc).
- II) **Indirect (serologic) method**: which depends on detection of antibodies against the microorganism in the patient's serum.
 ↳ from serum

I) DIRECT METHOD

1) Specimen Collection:

Direct method requires the collection of a 'good quality' clinical specimen from the patient. This is dependant on:

- a) Collecting specimens before the start of antibiotics.
- b) Choosing the appropriate specimen (representing the infection site).
- c) Using sterile containers and avoiding contaminating the specimen.
- d) Transporting the specimen properly to the lab. as early as possible.

Containers
sterile
even if stool
no bacteria

2) Microscopic Examination:

Specimens collected are usually examined microscopically before their further processing. Microscopic examination of stained or unstained (wet) preparations may be done using different types of microscopes

3) Microbial Detection:

Following microscopic examination, specimens are further processed for direct diagnosis by culture or non-culture techniques.

a) Culture technique: which includes procedures involving

- the **isolation** of the organism in pure culture (by inoculating the specimen onto appropriate artificial culture media), followed by
- **identification** of the isolate using different approaches, for example:

- ① microscopic examination
- ② biochemical reactions
- ③ reaction with specific antibody
- ④ DNA probes

(serologic identification of the organism)

Which of these approaches is used and in what sequence depends upon the type of specimen and organism.

After the organism is grown in pure culture, its sensitivity to various antibiotics is determined.

so after growth determine sensitivity to various antibiotics

not diagnosis

b) Non-culture technique: which includes procedures involving

- the identification of a **specific microbial antigen** such as a structural component (e.g. cell wall antigen, capsular polysaccharide...etc) or a product (e.g. an exotoxin) by reacting with specific antibody,

specific microbial antigen as
 ↳ ① structural component
 ↳ ② product
 ↳ ③ specific antibody



- or the identification of a **specific gene sequence** (i.e. nucleic acid of the organism) by the application of different molecular methods (e.g. PCR, DNA probe).

As these non-culture techniques do not depend on growth and multiplication of the organism, they yield more rapid results (minutes or hours); however antimicrobial susceptibility cannot be determined (although the presence of resistance genes can be determined by molecular methods).

* *What is Importance of Non Cultural direct? goods & bads?*

Non-culture techniques are mainly applied if:

- a rapid diagnosis is needed
- the microorganism cannot be cultured on artificial media
- the patient has received antimicrobial therapy
- the pathogen is a slowly growing organism

MCQ + enumerate

II) INDIRECT (SEROLOGIC) METHOD

* **Serologic diagnosis** of infectious diseases involves the use of known microbial antigens to detect **antibodies against the microorganism** in the patient's serum.

Detection of one of the following indicates current (active) infection:

- specific IgM antibodies
- rising titre of specific IgG antibodies (4-fold or greater rise)
- a single high titre of IgG antibodies in certain diseases

titre = Antibody LVL

N.B. **Skin tests** based on **cell-mediated hypersensitivity** may also help in the diagnosis of certain diseases. *rarely used*

Diagnosis of infectious diseases

DIRECT METHOD

Specimen (e.g. pus)

microscopical exam.

INDIRECT METHOD

(SEROLOGICAL) *diagnosis*

① Detection of antibodies in serum

- IgM
- rising titre of IgG

② *skin tests based on cell med. hypersensitivity*

A Culture technique

Isolation on culture media

Identification e.g.

- microscopical. ex.
- bioch. reactions
- DNA probe
- serology *Identification*

Antibiotic sensitivity

B Non-culture technique

- detection of specific antigen (serology)
- detection of specific gene sequence (mol. tech.)

good ① *Rapid diagnosis*

② *if microorganism not cultured*

③ *if patient received antimicrobial therapy*

④ *pathogen slow growing*

bad • *Antimicrobial susceptibility not determined*

Freely you have received; freely give.

- * microscope in Spirochetes? dark field
- * " used UV. Light? fluorescent
- * uses of microscopes

Microscopical Examination

Freely you have received; freely give.

الفرقة بين محل الطرشي
و محل ٢٥٥٠ و ٢٥٥٠ (٥٥٠)

mination



Microscopical Examination

Microorganisms are so small that they can not be seen by the naked eye. For this reason, different types of microscopes have been developed. Microscopical examination allows us to study the morphology of bacteria which is the first step in identification. Morphological study includes: size, shape (e.g. cocci, bacilli, vibrios), arrangement (e.g. clusters, chains, pairs), motility and staining characteristics.

Morphology = ① size ② shape ③ arrangement ④ motility ⑤ staining

Microscopes



A- Light microscope

Magnifications:

a- Eye piece x 10

b- Objectives:

- Low power $\times 10$ (final magnification: 100).
- High power $\times 40$ (final magnification: 400).
- Oil emersion lens $\times 100$ (final magnification: 1000).

$\rightarrow$ plain mirror

Uses:

- Uses:**
1. To study the morphology of bacteria: In this case, we use stained preparations and study it by the oil emersion lens with a final magnification of 1000, so a micron will be magnified to a millimeter.
 2. To study the motility of bacteria: In this case, we use a fresh unstained preparation (hanging drop preparation), and examination is done by the low and high power of the microscope.

In case of the oil emersion lens, we use the plane mirror, otherwise we use the concave mirror.

40000 L.E ← 4 months

B- Dark-field (dark-ground) microscope used to study unstained preparations of spirochaetes which can not be seen by the light microscope. In this type of microscopes the light is directed obliquely, by a special condenser, so that it can not pass through the lenses unless reflected by the spirochaetes which will appear as motile illuminated spiral bodies against a dark background.

 **C- Fluorescent microscope (ultraviolet microscope)** in which we use dyes which give fluorescence on exposure to ultraviolet light.

Uses:

- Uses:**
1. To study the morphology of bacteria by staining them with a fluorescent dye, e.g. auramin-O to stain tubercle bacilli (if they are very few in number).
 2. Immunofluorescence (direct and indirect).

2. Immunofluorescence **direct** and **indirect**

McAb

visible ← wavelength → 96 elongation hai circle ← U.V. not visible
Auramin-O stain T.B.



D- Electron microscope in which illumination is obtained by an electron beam (with very short wave length). The electron beam passes through an evacuated column and is focused on the object by means of electromagnetic condenser. Electron microscope has a magnifying power of 100,000 or more.

Uses:

1. To study the ultra-structure of bacteria and tissue cells.
2. To study the structure of viruses and rickettsia.

McQ
Light 1000 times

E- Phase contrast microscope is used to study the internal structure of the cells, e.g. nuclear body. By the use of special condensers and objectives, light is diffracted by any structure in the cytoplasm whose density is higher than the surrounding medium.

Light

① Simple
② Differential
③ Special

Staining Characteristics of Bacteria

3 Stains

There are many techniques for staining bacteria. The most commonly used are simple staining techniques and differential staining techniques. Special staining may be indicated, e.g. Fontana stain for spirochaetes, Giemsa stain for rickettsia and auramin O stain for T.B.

- a. Simple stains** in which one dye is used, e.g. methylene blue, crystal violet and safranin. All bacteria stained will take the colour of the dye.
- b. Differential stains** in which two dyes, a primary stain and a counterstain, separated by a decolourizing agent are used. The first dye is applied and then a decolourizing agent is used. Some bacteria are decolourized and others will retain the dye. When the second dye (counterstain) is applied, the bacterial cells which have been decolourized will take the colour of the counterstain.

The most commonly used differential stains are Gram stain and Ziehl-Neelsen stain.

N.B.: The steps of Gram's stain and Ziehl-Neelsen stain are illustrated. Before staining, a smear from the sample should be fixed on a clean glass slide.

In some disease conditions microscopical examination is sufficient for diagnosis:

- Acute gonorrhoea in male patients.
- Secondary cases of cholera during an epidemic.
- Leprosy.
- Vincent angina.
- Syphilis during the primary stage (chancre).
- Relapsing fever during the febrile stage.

Ex. 100
Enumerate

Ziehl Nelsen

Decolourizing agent T.B bacilli is

1% H₂SO₄

HCl in 95% EtOH

non of above

White Knight Love

Differential stain

Name of Person Gram's Stain

(1) Primary stain

Methyl violet + Gram's iodine

Decolourization by 95% ethyl alcohol

(2)

Not decolourized (Gram +ve)

Decolourized (Gram -ve)

ایجابی
عنه قراره الخالی
و ششایی

(3) Counterstain by dil. carbol fuchsin (Red)

Violet
peptidoglycan cell wall
hardly widen pores
of can't

Ziehl-Neelsen Stain

(1)

Strong carbol fuchsin + Heat (5-10 min)

more than 20% no clous

(2) Decolourization by H_2SO_4

- 20% in T.B.
- 5% in leprosy
- 0.5% for spores
- 1% for Nocardia

Not-decolourized (Acid-fast)

Decolourized (Non acid-fast)

(3) Counterstain by methylene blue

Red

Blue

N.B.: Decolourization can be done by 3% HCl in alcohol.

Why methyl violet & Iodine not in one step?

- All wall has pores
- Methyl violet entire
- Iodine + Methylviolet = Complex
- largest than pores
- So if was in one step Complex formed not entire
- not fixed

any other organ will give blue

only TB
Leprosy
spores
Nocardia appear Red

* TB isn't stained by Gram stain why
Lipid Like wax Can't be stained

G.R. after which you prefer Solid

Culture of Bacteria

fluid media
solid media

The clinical specimen is inoculated on carefully selected culture media to provide the optimal conditions for growth and multiplication of bacteria.

Media for Bacterial Growth

According to its physical state, media may be liquid or solid. Solid media are preferable because:

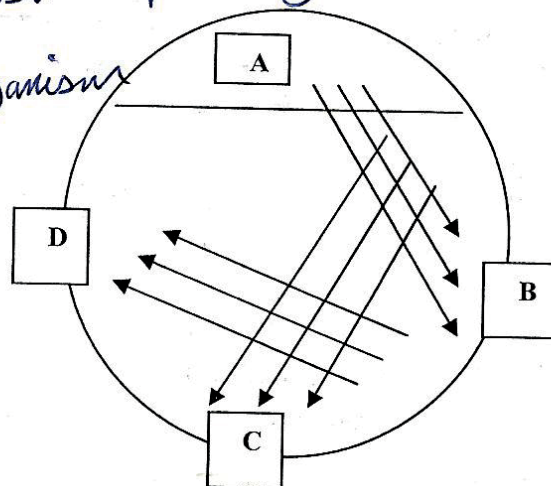
1) Solid media allow isolation of the organism present in a mixture in **pure culture** for further studies. This can be achieved by **the plating out technique**: (Fig.9)

- Using a sterile loop, the bacterial inoculum (e.g. in a clinical specimen) is smeared over area A to give a 'well inoculum' (or main inoculum).
- The loop is resterilized by flaming, cooled, and 3 parallel lines (B) are drawn from the well on to the surface of the medium.
- This process is repeated (lines C and D) with sterilizing and cooling the loop between each sequence.
- This technique ensures that bacteria from the infective material are ultimately deposited singly and at sufficient distance from each other so that the whole progeny of each single bacterium accumulates locally during growth to form a discrete mass, or **colony**, seen by the naked eye. Each colony is presumed to be a pure culture.

2) Solid media help in **identification** of the organism (different organisms have different colonial morphology and different effects on various media).

① isolation organism in pure form

② Identification of organisms



selective agents

renders

① malachite green dye

② potassium tellurite

③ antibiotic

Fig. (9): Plating out technique

* Nutrient agar → Transparent

* Blood agar → not transparent = opaque

How to sterilize

99

nutrient agar sterile in autoclave
melted then cooled → at 50%
to autoclave



support growth of most bacteria.

Important Culture Media

Autoclav function
sterile culture media



A- Simple (basal) Media

1. **Nutrient broth:** Meat extract or infusion
2. **Peptone water:** Water soluble products obtained from protein materials digested by proteolytic enzymes like trypsin or pepsin. The important ingredients are peptones, proteases and amino acids.
3. **Nutrient agar:** It is nutrient broth solidified by addition of 2% agar-agar. Agar-agar has no nutritive value. Peptone water, nutrient broth and nutrient agar are sterilized by autoclaving.

GR. why nutrient broth & agar have same nutritive value?

B- Enriched media: They are prepared to meet the nutritional requirements of fastidious or metabolically exacting bacteria. This is done by the addition of blood, serum, yeast extract or egg to a basal medium.

1. **Blood agar** is prepared by adding 5-10% blood (sheep, horse or human), collected under aseptic precautions on anticoagulant, to sterile nutrient agar melted and cooled to 50°C. In addition to being an enriched medium, it is an indicator medium showing the haemolytic properties of bacteria e.g. **streptococci**, may cause complete (β) partial (α) or no haemolysis. So can be said to be differential.

2. **Heated blood agar (chocolate agar)** is prepared as blood agar medium then reheated to 98°C. The heat ruptures the red cells and changes haemoglobin to haematin which is chocolate brown in colour. This medium is suitable for cultivation of **Haemophilus** and **Neisseria**.

3. **Löffler's serum medium** consists of serum (horse, ox, ... etc) and glucose broth in a ratio of 3:1. The medium is rendered solid by inspissation in tubes held in a sloped position at a temperature 75-85°C for 1 hour. At this temperature the serum protein is completely solidified. The medium is suitable for growth of **C. diphtheriae**.

C- Selective media contain substance(s) that inhibit the growth of some organisms but have little or no effect on the organism of interest. These inhibitory substances may be dyes, chemicals or antibiotics, e.g.:

1. **Lowenstein Jensen medium** consists of beaten eggs, mineral salts and is rendered selective by addition of malachite green dye. The medium is solidified by inspissation and dispensed in screw-capped bottles. The medium is selective for growth of **M. tuberculosis**.

2. **Blood tellurite** is a blood agar rendered selective by addition of potassium tellurite (0.03%). The medium is used for isolation of **C. diphtheriae**.

3. **Modified Thayer-Martin medium (MTM)** is a chocolate agar supplemented with special growth factors required for pathogenic **Neisseria** and rendered selective by adding certain antibiotics.

D- Enrichment media are liquid media with selective properties, i.e. favour the multiplication of a particular species and suppress competitors as a step towards their isolation in pure culture.

1. **Selenite F-broth** is probably the most used enrichment medium for isolation of **salmonella** and **shigella** from stools.

2. **Tetrathionate broth** may also be used to isolate **salmonella** from stools.

agar 2%
agar is
no nutritive
value
only
solidifying
agent

serum: Glu
3 : 1
↓
inspissation
in tube
in
slope
75-85°C
for 1h

become why
solid
with
no agar
agar
↓
denaturation
+
coagulation
of ptn

* mention enriched factor in each media



3. **Alkaline peptone water (pH 8.6)** is a useful medium for isolation of *V. cholerae* from faeces and contaminated materials.

E- Differential (indicator) media contain some substances that are changed visibly as a result of metabolic activity of a particular organism.

1. **Sugar media:** A sugar medium is composed of peptone water, 1% of the test sugar and a pH indicator. Sugar media are used in the identification of bacterial species by the pattern of their fermentative activities on different sugars.

2. **Triple sugar iron (TSI) agar:** This medium is composed of 0.1% glucose, 1% lactose, 1% sucrose, ferrous sulphate for detection of H_2S production, phenol red (pH indicator), beef extract and yeast extract.

The medium is solidified by 0.5% agar (soft agar that cracks easily on gas production). *not 2% why?*

The medium is poured in test tubes in the form of a slant and a deep butt. Sterile uninoculated medium is transparent red in colour. The tubes are inoculated with the isolated organism and incubated at $37^\circ C$ for 24 hours.

• Bacteria that ferment glucose only (non lactose fermenter) give red slant and yellow butt, e.g. **salmonella and shigella**.

• Bacteria that ferment glucose, lactose and sucrose give yellow slant and butt, with production of gas which causes the agar to crack, e.g. **E. coli**.

• Bacteria that ferment glucose and produce H_2S give blackening of the butt, e.g. **proteus**.

• Bacteria with no fermentative activity give red slant and red butt e.g. **Pseudomonas aeruginosa**.

+ differential

Butt Slant

F- Selective and indicator media: Combination of selective and indicator supplements in basal media restricts growth to a desired range of organisms and can differentiate between them:

1. **MacConkey's agar** contains bile salts to inhibit non intestinal bacteria, and lactose (a test sugar) with neutral red (pH indicator). The medium is used to distinguish lactose fermenting coliforms (give pink colonies) from lactose nonfermenting salmonella and shigella (give pale colonies).

2. **Deoxycholate Citrate Agar (DCA)** contains bile salts, citrate and other substances to inhibit intestinal coliforms. It is superior to MacConkey's medium in isolation of salmonella and shigella.

3. **TCBS**

• The medium contains thiosulphate, citrate and bile salt (selective substances), sucrose (test sugar) and bromothymol blue (pH indicator).

• The medium is used for isolation of *V. cholerae* which produces yellow colonies due to fermentation of sucrose and production of acid which changes the colour of the indicator into yellow.

** all are selective factors in TCBS except thiosulphate, bile salt, citrate, alkalinity*

Anaerobic Culture

$EH = \text{Oxidation Reduct potential}$

Obligate anaerobes will not grow in culture unless oxygen is absent and the Eh of the medium is low. This can be achieved either by exclusion of oxygen or by the addition of reducing substances to culture media. Freshly steamed liquid media are temporarily anaerobic. They soon become aerobic unless a reducing agent is added.

*SUCROSE
→ differentiator X
but not selective*

Differential media

= Indicator

• sugar media • TCI
• 101 TCBS • MacConkey

*only for
Bloo agar
Lofler serum*



The most commonly used anaerobic **liquid media** are:

1. **Thioglycollate broth** in which 0.1% sodium thioglycollate is added as a reducing agent.
2. **Cooked meat broth** in which cooked minced meat is added. The meat contains haematin, glutathione and ascorbic acid which act as reducing agents.

v.t.c

Gaspak system:

For the routine surface culture of anaerobes of medical importance, freshly poured blood agar plates are recommended. After inoculation with the clinical specimen, the plate is placed in a special jar (**Gaspak system**). In this system, hydrogen is generated inside the jar from commercially available Gaspak envelope on addition of 10 ml distilled water. The jar contains palladium catalyst which allows the released hydrogen to combine with the oxygen inside the jar. The tightly fitting lid of the jar prevents air from entering inside. It is recommended that 10% CO₂ to be present in the anaerobic atmosphere as CO₂ enhances the growth of many clinically important anaerobes on solid media.

3. *deep agar medium example for anaerobic culture*

special → **Sample blood** → **Blood Culture** (not culture on Blood agar) *ONV, 12*

Blood culture is required whenever bacteraemia (or septicaemia) due to any organism is suspected. In most cases of bacteraemia, the organisms are not numerous in the blood and, so, direct plating of a drop of the patient's blood will usually give a negative result. Therefore, for diagnosis of such infections, the **blood culture technique** is applied as follows:

sample on culture Blood agar

- Large volume of blood (5-10 ml) is withdrawn under strict aseptic conditions.
- The sample is added to 50-100 ml broth.
- The inoculated broth is incubated aerobically and anaerobically at 37°C.
- Subcultures are done on suitable solid media every 48 hours and continued for up to 10 days before the sample is discarded as negative.
- Growing organisms are identified in a systematic way.

G.R. Why large volume broth? → dilute host factor reduce growth which not grow into bacteria grow

To increase the chance of isolating a pathogen, 3 blood culture sets are done. These should be taken from different sites and collected at different times.

The large volume of broth used in blood culture has the following advantages:

1. It reduces the concentration of natural antimicrobial constituents in the blood to a sub-inhibitory level.
2. It allows the growth and multiplication of the small number of organisms present in the blood sample.
3. In patients under antibiotic therapy, neutralization or diminution of antibiotics in blood may be done by different methods.

Common infectious diseases diagnosed by blood culture :

- Enteric fever
- Brucellosis
- Endocarditis
- Meningitis
- Puerperal sepsis

مؤهلين

Pure separate Colony



Biochemical Reactions

12

In some organisms, the colony morphology (shape, size, pigment production, consistency ... etc) together with the microscopical morphology of the organism may be sufficient for diagnosis, e.g.

- a- Gram negative, motile bacilli showing greenish blue exopigment on nutrient agar is *Pseudomonas aeruginosa*.
- b- Gram negative, motile bacilli showing swarming growth on nutrient agar is *proteus*.

→ In many other conditions, it may be necessary to proceed for further diagnostic procedures.

Bacteria vary in their metabolic and enzymatic activities. This can be used in identification of different genera and species of bacteria. For this reason, **one or more** of the following biochemical reactions may be done on bacteria grown in **pure culture**.

1. Sugar fermentation tests: Sugar media are composed of peptone water (1% test sugar) (glucose, lactose, maltose, mannite, sucrose) and Andrade's indicator which becomes pink in acidic pH. A small inverted tube (Durham's tube) is placed in the medium to detect gas production. *Andrade indicator pink acid + gas production Durham tube inverted*

Bacteria vary in their fermentative activities on different sugars. They produce either acid only or acid and gas.

2. Indole test: Some bacteria (e.g. *E. coli* and *Vibrio cholerae*) can act on tryptophane (an amino acid present in peptone water) with production of free indole. This can be demonstrated by addition of Erlich's or Kovac's reagent which gives a red ring (indole +ve). A negative test gives a yellow ring. *tryptophane → indole → red ring*

3. Methyl red (M.R.) test: Some bacteria (e.g. *E. coli*) when grown on glucose phosphate peptone can produce large amount of acid (from glucose fermentation) so that the pH of the medium is reduced below 4 and the colour of the M.R. indicator becomes red (M.R. +ve). A negative test gives a yellow colour. *both together*

4. Voges-Proskauer (V.P.) test: Some bacteria (e.g. *Klebsiella pneumoniae*) when grown on glucose phosphate peptone produce acetyl methyl carbinol from glucose fermentation. The production of acetyl methyl carbinol can be detected by addition of concentrated KOH which gives a pink color (V.P. +ve). A negative test gives a yellow colour. *+ve MR = -ve VP V. Verse*

5. Citrate utilization test: Some bacteria (e.g. *Klebsiella pneumoniae*) are able to use citrate as their only source of carbon. The test may be performed by inoculation of the organism in either Koser's citrate medium (liquid), where turbidity indicates bacterial growth (citrate +ve), or in Simmon's citrate medium (solid), where the original green colour of the medium turns into bright blue colour (citrate +ve). *turbid bright blue colour*

6. Urease test: Some organisms (e.g. *proteus*) produce urease enzyme and, so, if grown on urea agar containing phenol red indicator, ammonia is released and the medium turns red (urease +ve). *-ve yellow alkaline*

red → acid < 4 pH > 4
MR +ve *E. coli* → ferment glucose

- a.sulphur + lead acetate → lead sulphide black filter paper

7. **H₂S production:** Some bacteria (e.g. *Salmonella typhi*), if grown in peptone water can decompose the sulphur-containing amino acids present in the medium and form H₂S. This can be tested for by a strip of filter paper saturated with lead acetate which turns black due to formation of lead sulphide (H₂S +ve)

black filter

8. **Oxidase test:** Some bacteria (e.g. *neisseria* and *pseudomonas*) produce oxidase enzyme. If cultures of such bacteria are treated with oxidase reagent (tetramethyl-p-phenylene diamine hydrochloride), a deep purple colour is produced within few seconds (oxidase +ve).

9. **Catalase test:** Some bacteria (e.g. *Staph. aureus*) produce catalase enzyme. If a colony of such bacteria is added to a drop of hydrogen peroxide, O₂ bubbles will be released immediately (catalase +ve).

+ve Bubbles

H₂O₂

10. **Coagulase test:** *Staph. aureus* produce coagulase enzyme (free and bound). Free coagulase (which changes fibrinogen into fibrin) can be detected within few hours by mixing the organism with human or rabbit plasma, to which an anticoagulant was added, in a test tube. It coagulates plasma by converting fibrinogen to fibrin leading to aggregation of the organism within the clot.

Presence of the **clumping factor** (bound coagulase), which binds with fibrinogen leading also to aggregation of the organism, can be detected within few seconds by mixing one drop of the bacterial suspension with one drop of human plasma, to which an anticoagulant was added, on a slide.

Commercial API Kit Systems (Analytical Profile Index)

Packaged biochemical identification systems are available from commercial sources. It consists of a plastic strip containing a series of small cupules, each containing dehydrated media and reagents. A suspension of the test organism is dropped in the hole at the top of the cupule and incubated at 37°C for 24-48 hours. The biochemical profiles are determined and interpreted according to the charts given with the kits.

Serological Identification of Bacteria

Bacteria have different antigens which correspond to different structures of the cell, e.g. somatic or "O" antigen, flagellar or "H" antigen and capsular antigens. An unknown bacterium can be identified by demonstrating its reaction with a specific antiserum (antibodies) using different antigen antibody reactions, e.g. agglutination, precipitation, immobilization, capsular swelling reactions....etc

Biochemical Reactions		
3	Sugar	→ → → sugar fermentation MR VP
2	a.a	→ → indole H ₂ S
1	others	→ Citrate
4	enzymes	→ → → 104 mention urease Catalase Coagulase substrates

organisms

	i	M	V	C
<i>E. coli</i>	+	+	+	-
<i>Klebsiella</i>	-	-	+	+

i = indole
M = MR = Methyl red
V = VP = Voges Proskauer

Bacterial Typing

A given species of bacteria can be classified into several types. Typing or 'fingerprinting' of individual strains of an organism is a major factor in epidemiological investigations, such as tracing the source of infection in an outbreak of post-operative wound sepsis or an outbreak of food poisoning. The bacterial strain isolated from clinical specimens obtained from the patients should be of the same type as that isolated from the suspected source(s).

Different typing methods are available for different organisms. Examples include: typing by colony morphology, bacteriophage-typing, biotyping, pyocin-typing, serotyping, plasmid analysis, ribotyping, chromosomal analysis and others.

Bacteriophage typing of *Staph. aureus* is done as follows:

The strain to be tested is inoculated on the surface of a suitable solid medium and left to dry. From each phage, a drop is placed in a marked square area on the surface of the plate and incubated at 30°C. After 24 hours incubation, a homogeneous growth of bacteria will appear on the surface of the plate except for areas of lysis denoting susceptibility to this phage. These areas will determine the phage type of the strain.

Molecular Methods for Diagnosis

Each bacterial species contains a **specific** DNA that is unique to it, thus DNA can be used for identification much like a fingerprint.

Detection of pathogenic organisms, by molecular technology is likely to be most useful when applied to those which are:

1. Difficult or costly to culture, e.g. *M. leprae*, HCV
2. Slow growing, e.g. *M. tuberculosis*
3. Present in low concentration in clinical samples, e.g. HPV
4. Hazardous to propagate in the laboratory, e.g. HIV

Identification A- DNA Probes

- specific
- single strand
- short sequence
- complementary
- labeled

- DNA probes are used for the detection of target (e.g. microbial) DNA by hybridization technique.
- A probe is a short segment of single-stranded DNA (ssDNA), consisting of a known sequence of nucleotides unique (specific) and complementary to the target DNA.
- Probes are labelled (e.g. by a radioactive isotope or an enzyme), so that they can be located later.
- They are usually prepared synthetically by DNA-synthesizing machines.
- The steps of hybridization technique include: (Fig 10)
 1. Extraction of target DNA, which may be present in a clinical sample or culture.
 2. Exposure of target DNA to heat or alkali to separate the 2 strands giving complementary ssDNA.
 3. Immobilization of the ssDNA on nitrocellulose membrane. *fixation*
 4. Addition of specific labelled probe.

Handwritten notes:
 Run on line
 Smear
 U.I
 light

5. Temperature and pH are returned to normal to allow the ssDNA to anneal (hybridize) to the complementary probe forming labelled double-stranded DNA (dsDNA). Excess probe should be added to allow ssDNA to anneal with probe rather than the unlabelled complementary strand.
6. Washing is done to remove unhybridized probe.
7. Detection of hybridized probe is then done by autoradiography or enzymatic assay, depending on the particular label it carries

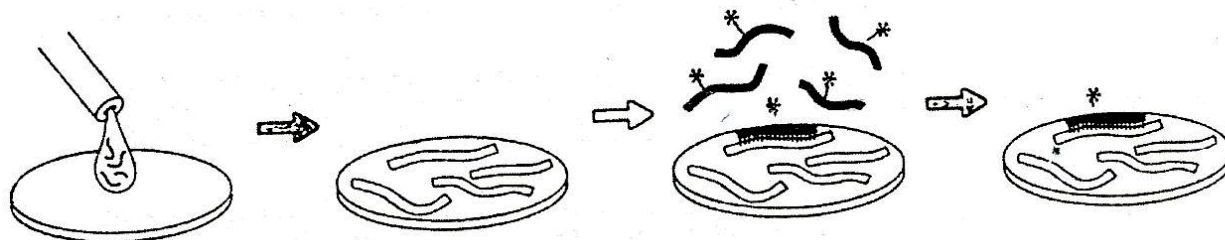


Fig. (10): A simple hybridization assay using nucleic acid probes.

amplifying by making copy **B- Polymerase Chain Reaction (PCR)** *only by heat*

PCR is used to amplify a particular region of target DNA selectively *in vitro*. It is a very sensitive technique that allows the detection of less than 10 copies of DNA in a sample by making billions of copies in only few hours. PCR is based on the repetitive cycling of 3 temperature-controlled steps (Fig 11):

1. **Denaturation** of extracted dsDNA in the sample to give ssDNA by applying high temperature. *not labeled* → *specific*

2. **Primer Annealing**

Two primers are used. These are two short oligonucleotides flanking the desired region of target DNA to be amplified. Their sequences are complementary to those on the flanking region on the 2 opposite strands. The temperature is lowered to allow denatured ssDNA to anneal with the primers. Excess of primers is necessary to avoid reannealing of the dissociated ssDNA to their corresponding dsDNA. *complementary to flanks*

3. **Primer Extension**

+ 2 primer + *pool*
Thermostable **Taq polymerase** and **nucleotide bases** are added. The enzyme catalyses primer extension by adding new nucleotides sequentially to the annealed primers producing 2 new complementary strands. Thus, after 1 cycle 2 new dsDNA strands are produced. With each subsequent cycle, the amount of amplified DNA is doubled, i.e. the process proceeds exponentially until the supply of primers is exhausted.

The amplified DNA product may be detected by:

1. Gel electrophoresis, which permits DNA fragments to be separated on the basis of their size; the smaller the fragments, the more rapid the rate of migration. A dye (ethidium bromide) binds to the separated DNA fragments and renders them visible by U.V. rays.
2. Hybridization with specific labelled probes.
3. Nucleic acid sequencing.

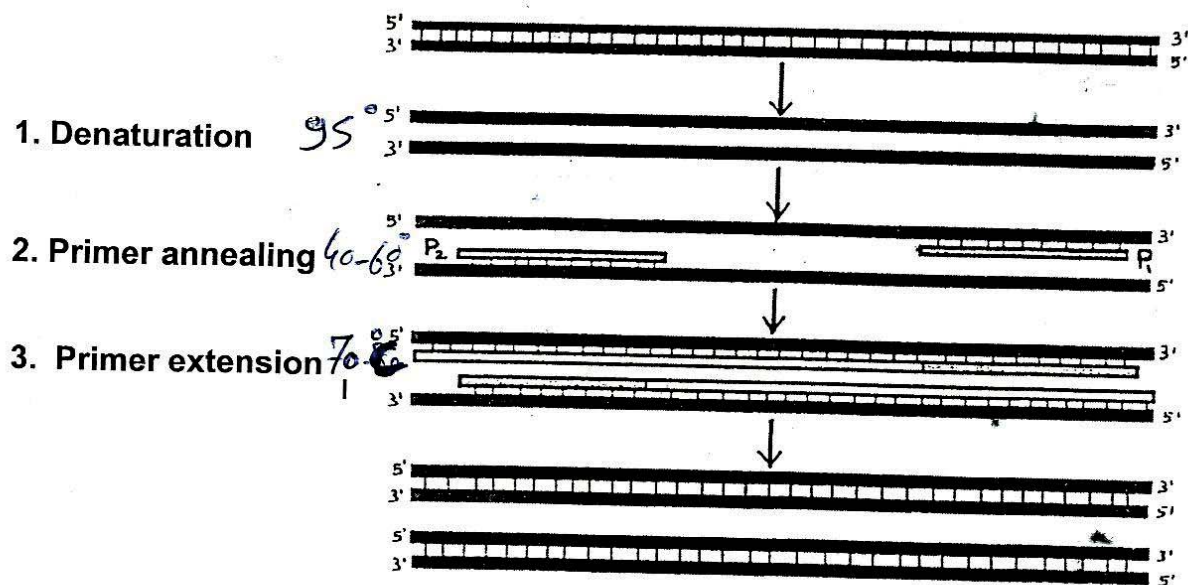


Fig. (11) : Diagrammatic presentation of the steps of PCR
P1 : primer 1 P2 : primer 2

C- Reverse Transcriptase PCR (RT-PCR) *from retro virus*

RT-PCR is used for the amplification of **RNA**. It requires 2 steps before starting PCR procedures:

1. Formation of ssDNA complementary to the target RNA by the enzyme reverse transcriptase.
2. Formation of dsDNA by the enzyme DNA polymerase.

D- Chromosomal DNA Restriction Endonuclease Analysis

This technique uses restriction enzymes, a class of bacterial enzymes that cut both strands of linear DNA molecule at specific short recognition sequences. This results in the formation of restriction DNA fragments, whose number and lengths are determined by the nucleotide sequence of the original cleaved (digested) DNA. Thus, if chromosomal DNA with different nucleotide sequences are subjected to digestion using the same restriction enzymes, this will lead to the production of DNA fragments of variable number and length; this is called **Restriction Fragment Length Polymorphism (RFLPs)**. The resulting restriction fragments are then subjected to gel electrophoresis which separates them according to length. The fragments are visualized by UV light after staining with ethidium bromide. Alternatively, the fragments may be transferred to nitrocellulose membrane and detected by hybridization technique using specific probes.

In Vitro Antibiotic Susceptibility Tests

Testing the sensitivity or resistance of pathogenic bacteria, isolated from a pathological specimen, to antibiotics is indicated for selection of the drug which is most suitable for treatment of the patient.

In vitro sensitivity testing can be done by one of 3 methods:

1. Disc diffusion method (Fig. 12):

Filter paper discs, each containing a standard amount of an antibiotic, are placed on the surface of a suitable solid medium that has been seeded with the test organism. After incubation, the diameter of the clear zone of inhibition surrounding the disc is taken as a measure of the inhibitory power of the drug against the organism. It is a semiquantitative, rapid and easy test for routine use.

2. Dilution method:

Serial dilutions of the drug are incorporated into a suitable liquid medium. The media are subsequently inoculated with the test organism and incubated. The highest dilution of the antibiotic which inhibited the growth of the test bacteria is referred to as the minimal inhibitory concentration (MIC).

→ *اقل تركيز يمنع النمو*
Turbidity

3. 'E' test (Fig. 13):

This is a new technique for measuring MIC. It has recently been developed for a direct quantification of antimicrobial susceptibility of microorganisms. A predefined, continuous, increasing gradient of antibiotic concentrations is immobilized along a rectangular plastic test strip. The strip is applied to the surface of a suitable medium inoculated with the test organism. After overnight incubation, a tear drop-shaped inhibition zone (elliptical) is seen. The zone edge intersects the graded test strip at the MIC of the antibiotic.

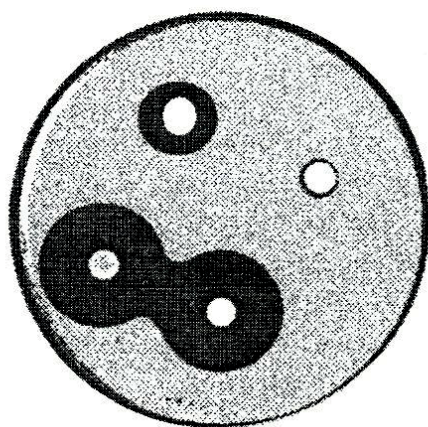


Fig. (12): Disc diffusion method.

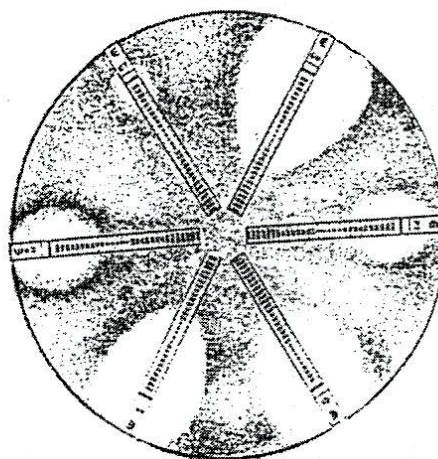
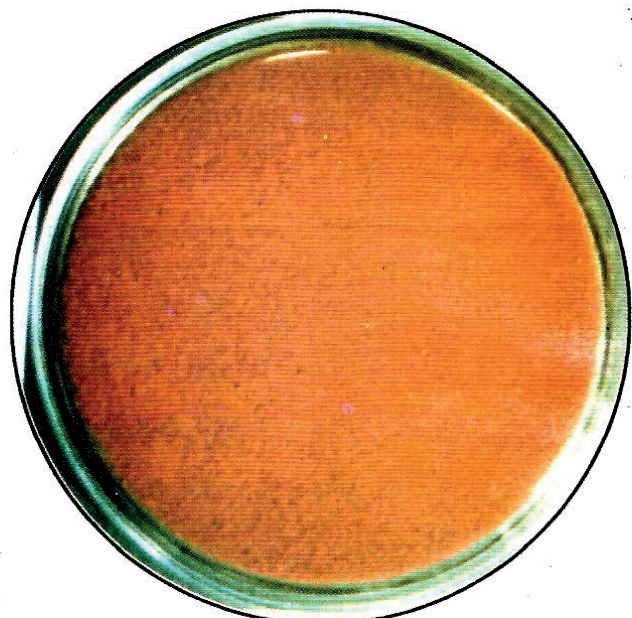


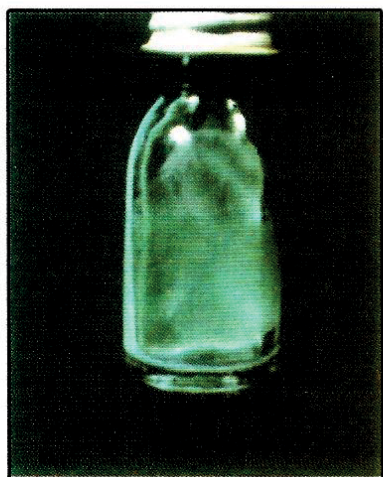
Fig. (13): "E test".



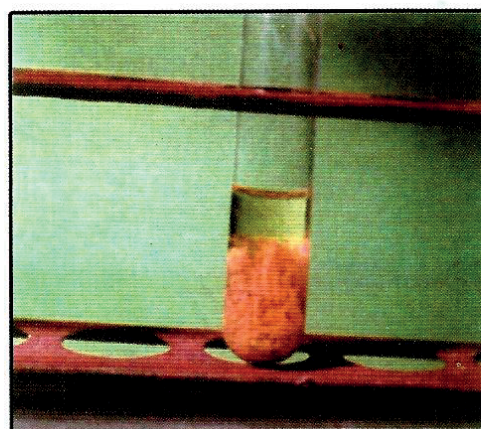
Chocolate agar



Löffler's serum



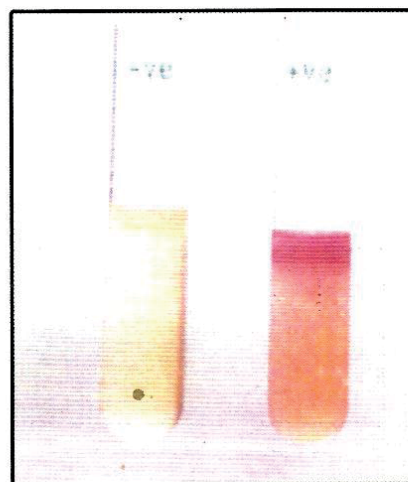
L-J medium (for T.B)



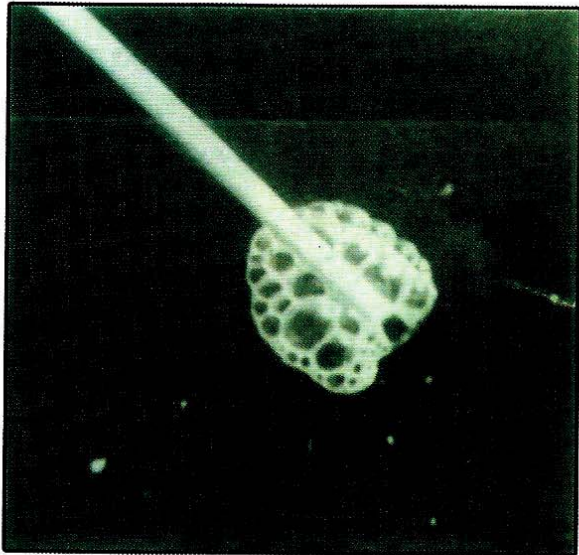
Cooked meat medium (for anaerobic culture)



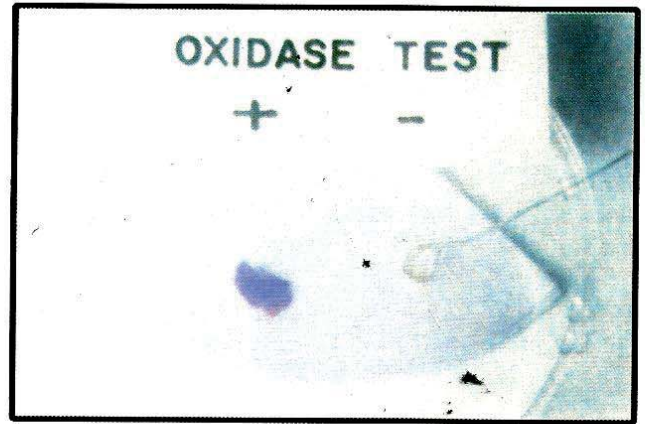
Gaspak system (anaerobic jar)



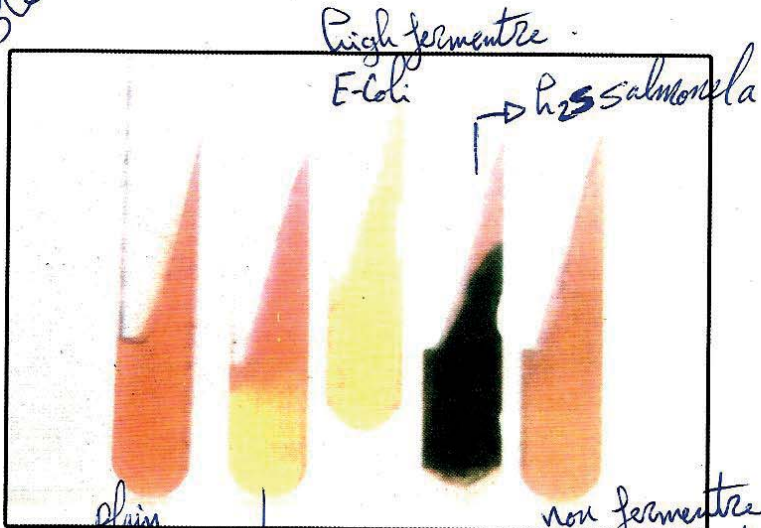
Indole test



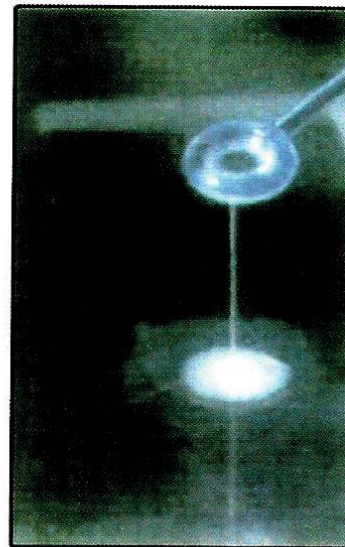
Catalase test



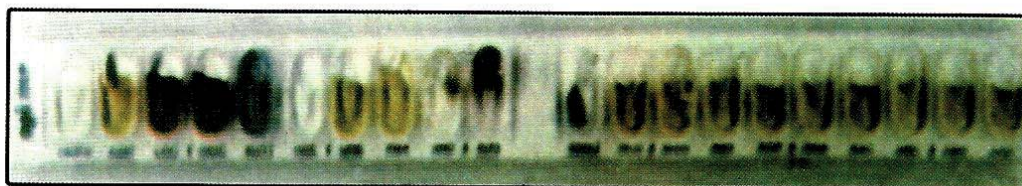
Oxidase test



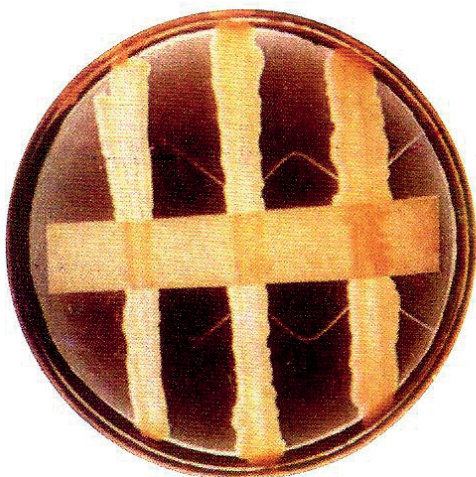
Effect of different bacteria on TSI agar



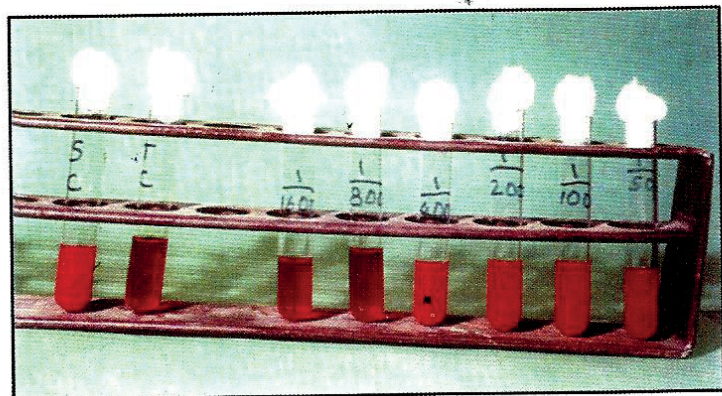
String test



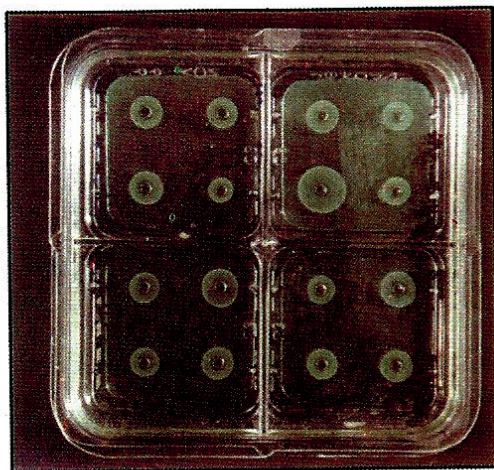
API-20E



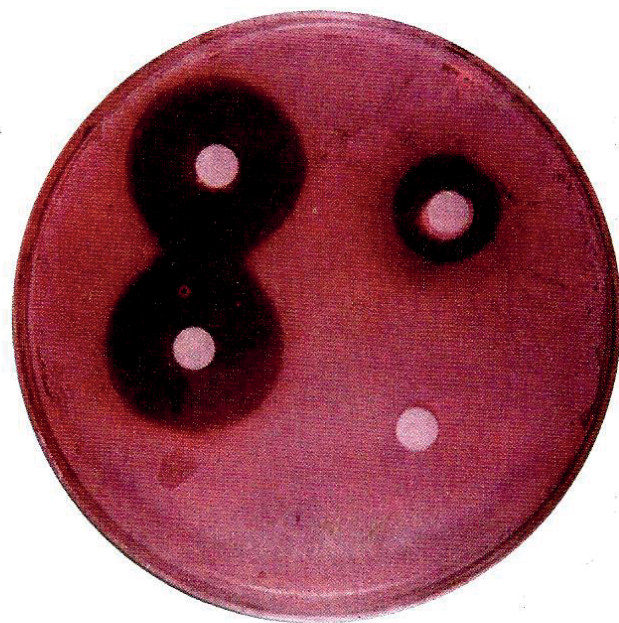
Elek's test



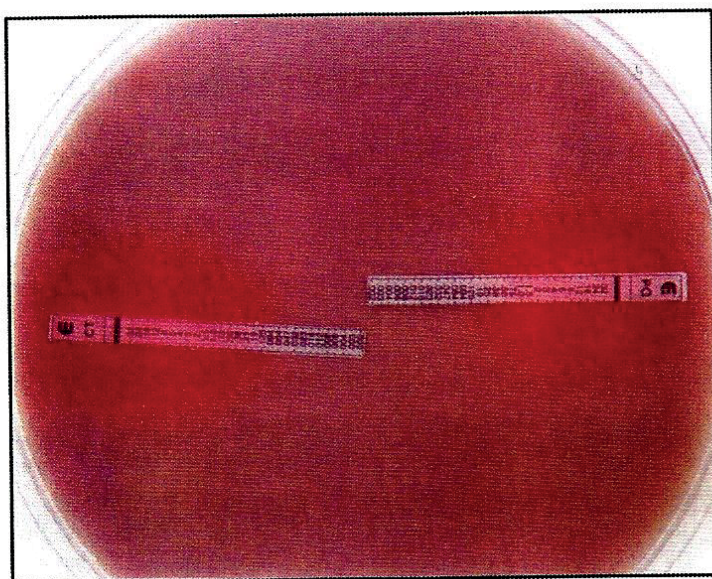
Antistreptolysin-O test (Titre 1/400)



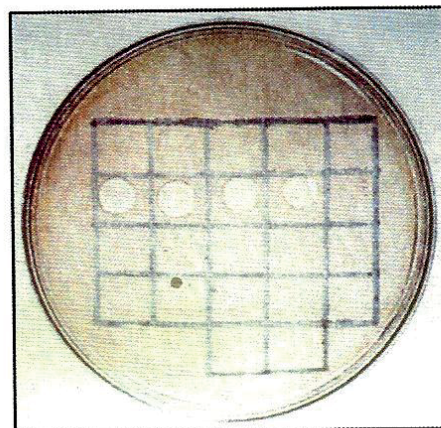
Single radial immunodiffusion



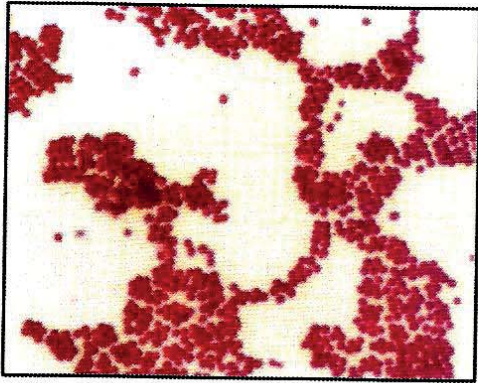
Antibiotic sensitivity test
(Disc diffusion method)



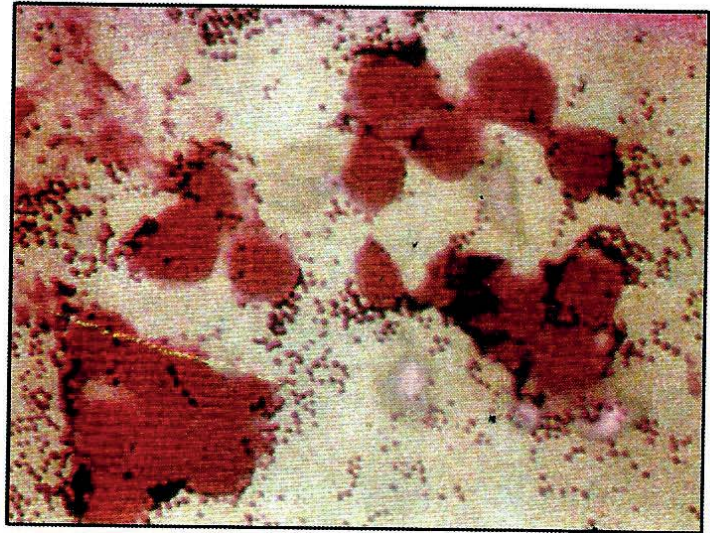
E-test for antibiotic sensitivity



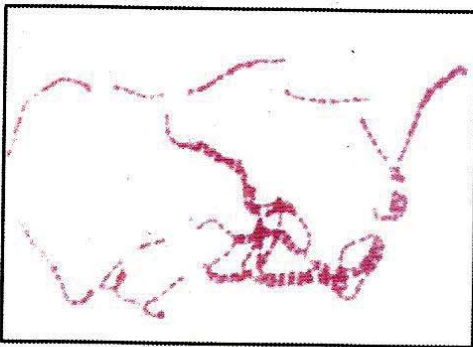
Bacteriophage typing



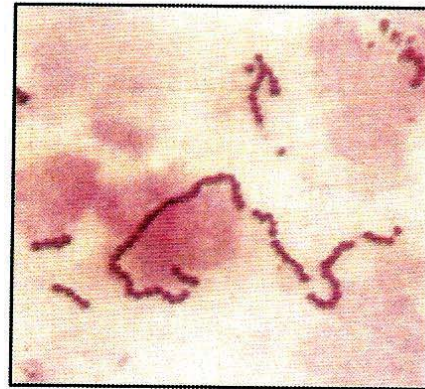
Staph. in culture



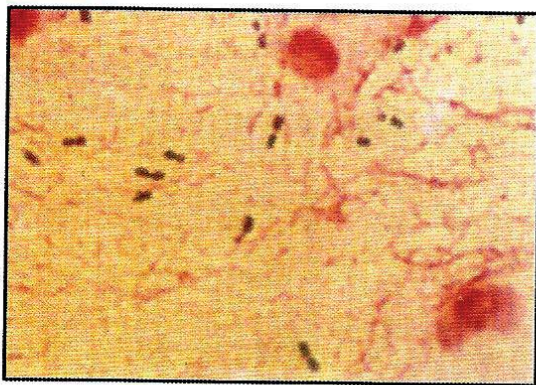
Staph. in pus



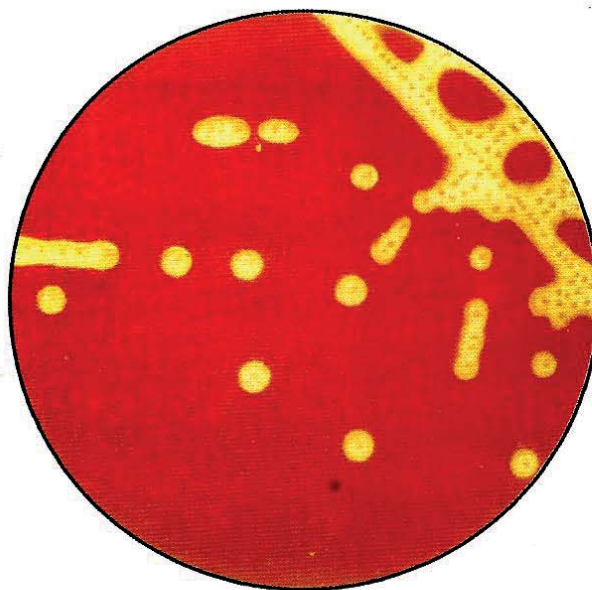
Strept. in culture



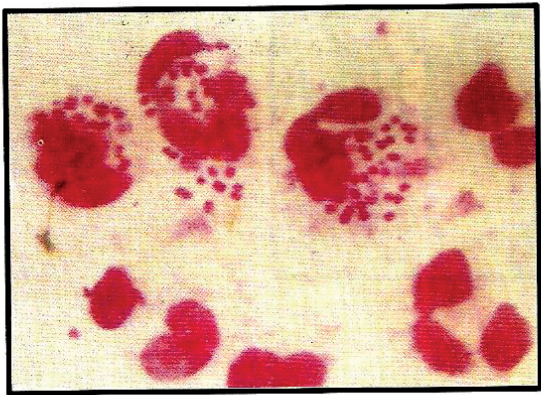
Strept. in pus



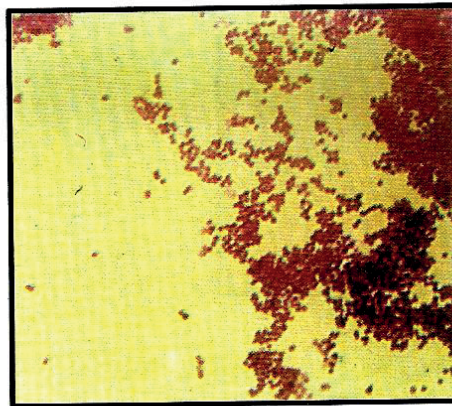
Pneumococci in tissue



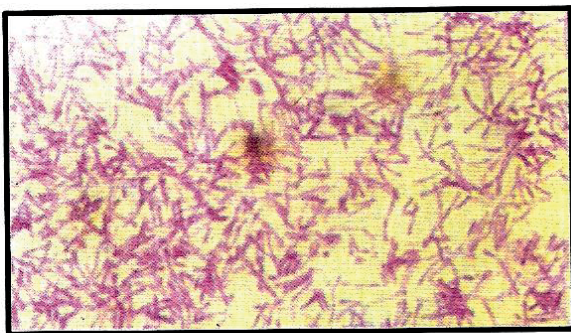
B-haemolytic organism on blood agar



Neisseria in pus



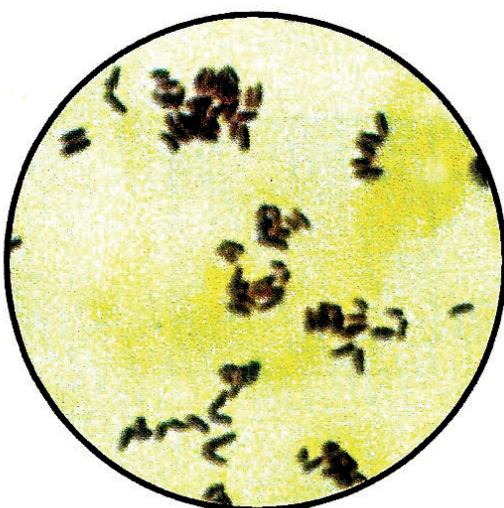
Neisseria in culture



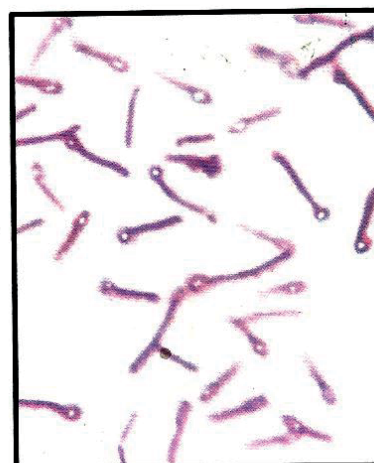
C. diphtheriae (Gram stain)



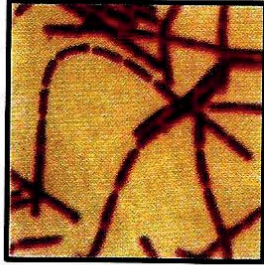
C. diphtheriae (M.B. stain)



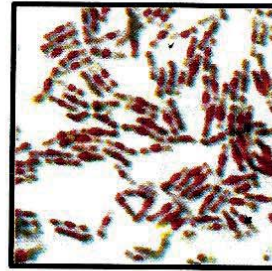
Diphtheroids



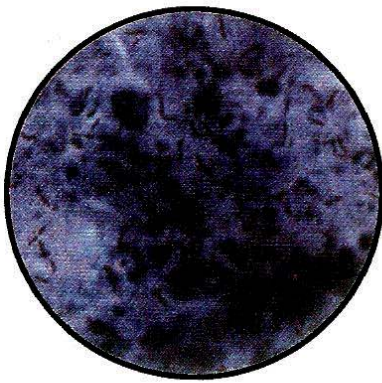
Clostridium tetani



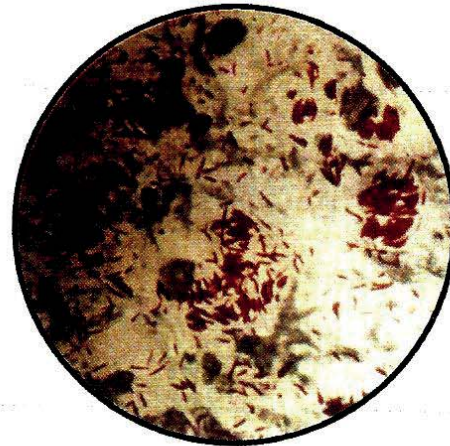
Anthracoids in culture (Gram stain)



Anthracoids in culture (spore stain)



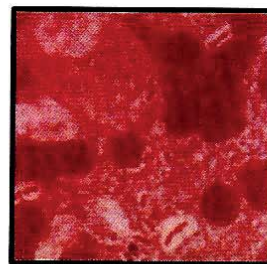
M. tuberculosis (Z.N. stain)



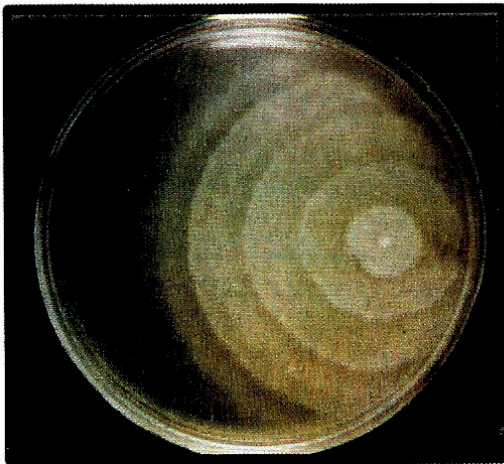
M. leprae (Modified Z.N. stain)



Gram negative bacilli



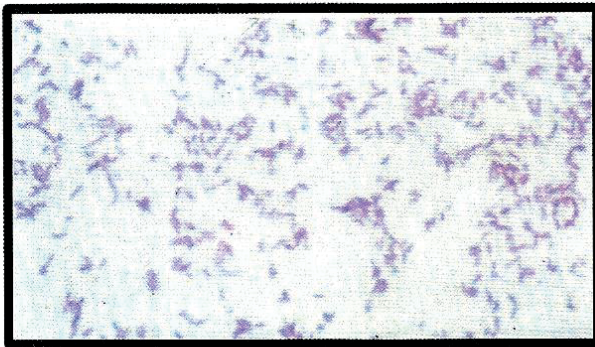
Klebsiella in tissue



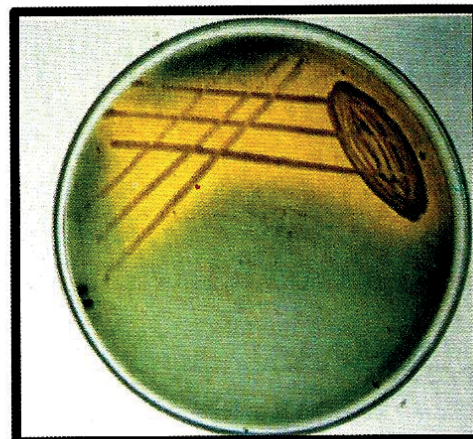
Proteus on nutrient agar
(swarming growth)



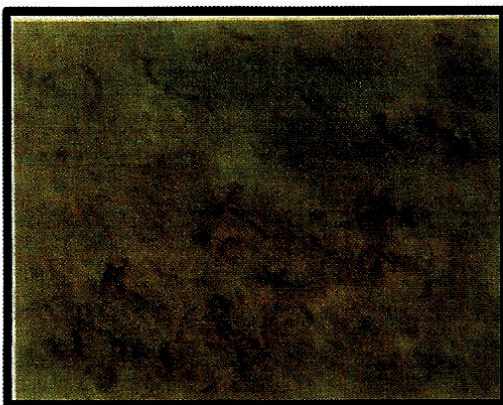
Pseudomonas exopigment on
nutrient agar



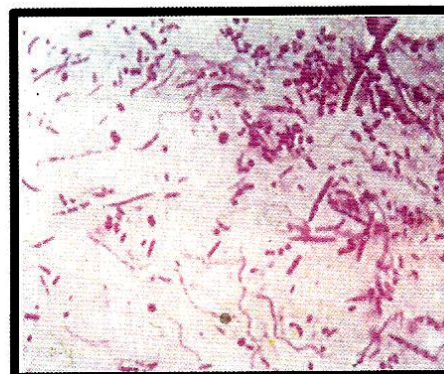
Vibrio



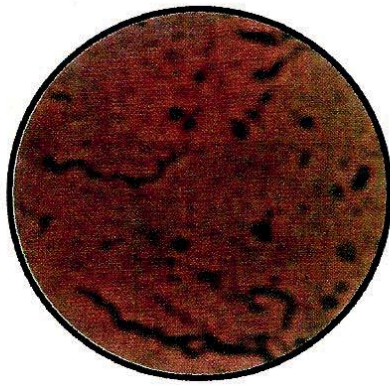
Vibrios on T.C.B.S.



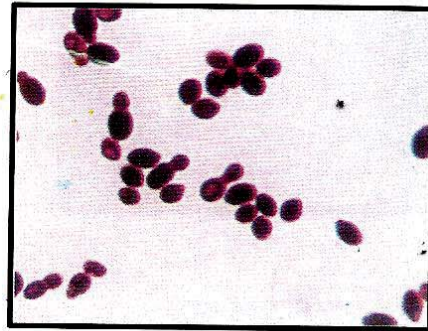
Helicobacter pylori



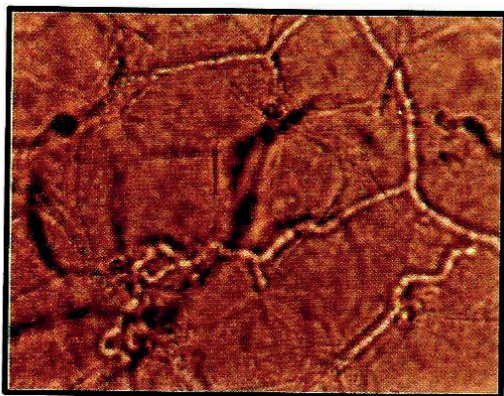
Vincent angina



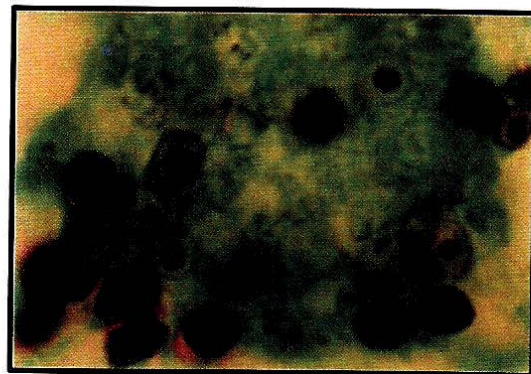
Spirochaetes (Fontana stain)



Candida (Gram stain)



Filamentous fungi (KOH mount)



Pneumocystis jirovecii (Silver stain)

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It is more blessed to give than to receive.